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Induction of T_1 (cytotoxic lymphocyte) and/or T_2 (antibody) responses to a mucin-1 tumour antigen

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Effective vaccination-based control of intracellular pathogens or parasites and various tumours is dependent upon induction of cytotoxic lymphocytes and other mechanisms of cellular immunity. Such responses are usually described as being antagonistic to an antibody-based immune response. This paper elaborates on previous studies that have demonstrated that conjugation of a fusion protein (FP, incorporating copies of the variable number of tandem repeat sequence of human mucin-1 (MUC1)) to oxidized mannan results in a significant shift from a type-2 response towards a type-1 response. This response induces complete protection upon challenge of immunized mice with MUC1 expressing tumour cells. This report details experiments in which the balance between type-1 and type-2 anti-MUC1 responses is manipulated by altering the dose of mannan–FP (M–FP) delivered. It is also shown that type-1 and type-2 responses may be induced simultaneously by administration of both forms of the antigen (FP/M–FP). Further, when a type-2 response is induced after FP immunization, a type-1 response can also be established by subsequent immunization with M–FP without adversely affecting the initial response. The converse also applies when M–FP is used for the initial immunizations, followed by FP administration. Delivery of interleukin-1β as a cytokine adjuvant with M–FP immunizations also enhanced antibody responses to levels fourfold that induced by M–FP alone without adversely affecting the cytotoxic activity induced by M–FP immunization. Contrary to the type-1/type-2 paradigm, cellular and antibody responses to MUC1 were not antagonistic. These results have important implications for the development of vaccination strategies against pathogens for which both the cellular and humoral compartments of the immune response contribute to protection. © 1997 Elsevier Science Ltd.

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The current model of immunological responses to vaccination or infection involves a segregation into cellular and humoral responses that have most often been described as antagonistic. While initial descriptions of such division of immune responses referred specifically to subsets of $CD4^+$ helper T cells (Th1/Th2)[1,2], recent work has shown that other T cell types may also be subdivided on the basis of their secreted cytokines and subsequent effector functions[3,4]. The type-1/type-2 model now more accurately aims to describe the total phenotype of the immune response. Type-1 responses are characterized by cellular effector functions including macrophage activation, cytotoxic activity and delayed type hypersensitivity (DTH), accompanied by the production of complement fixing and opsonizing antibody (IgG2a), in response to secretion of the cytokines interleukin-2 (IL-2), IL-12 and interferon gamma (IFNγ). Conversely, Type-2 responses are characterized by antibody responses (IgG and IgE), secreted under the influence of cytokines including IL-4, IL-5, IL-6 and IL-10. While in the case of some severe parasitic infections in mice[5,6] these immune phenotypes are well defined, and their effects result in either protection from, or exacerbation of disease effects, such a distinct division is not clearly recognized in most cases. The antagonistic nature of the cellular and humoral compartments of the immune response however, has long been recognized and remains a central theme in the definition of immune responses. As a result, vaccine research has adopted a 'rational' approach in which the most appropriate protective immune response is determined and strategies are developed to induce such a response.

Significant progress has been made in the development of immunization protocols capable of selective

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induction of type-1 responses[8–10], offering hope for the application of vaccines against diseases for which antibody mediated strategies have previously proved unsuccessful, including malaria, human immunodeficiency virus and various cancers. Apostolopoulos *et al.*[11,12] have demonstrated the effectiveness of conjugation of antigen to the carbohydrate mannan to induce type-1 responses against breast cancer. The target antigen is human mucin-1 (MUC1), a heavily glycosylated molecule that is expressed normally in breast tissue. In the case of breast cancer, MUC1 is overexpressed and exhibits reduced glycosylation so that the protein core of the molecule becomes exposed to immune surveillance[13,14]. The variable number of tandem repeat (VNTR) sequence of the protein core of MUC1 was selected as a target for breast cancer vaccination as it has been shown to be the most immunogenic fragment[15,16], and a glutathione-S-transferase (GST) fusion protein (FP) incorporating five repeats of the VNTR was tested as a candidate antigen. Immunization of mice with FP induces strong antibody responses with little cytotoxic activity and minimal protection on challenge of mice with MUC1 expressing tumour cells. A conjugate of mannan and FP (M–FP) however induces a startling shift in the immune response observed, provided that the conjugation process occurs under oxidizing conditions. Immunization with oxidized M–FP results in development of cytotoxic lymphocyte (CTL) activity, high CTL precursor (CTLp) frequencies, weak antibody responses and complete protection against challenge. Immunization with FP conjugated to mannan under reducing conditions induces responses similar to that induced by FP alone.

This report further analyses the type-1/type-2 division of the anti-M–FP (oxidized) response, and shows that the response may be manipulated by using varied doses of antigen. The simultaneous induction of type-1 and type-2 responses is also described using antigen selection or a cytokine adjuvant. The results show that although cellular or humoral responses against M–FP may be selectively induced, they are not mutually exclusive. Techniques described here may be extended to develop vaccination strategies where both antibody and cellular responses contribute to protection.

MATERIALS AND METHODS

Antigen preparation

The human MUC1 FP was composed of five repeats of the 20 amino acid VNTR sequence of human MUC1 and GST (PAHGVTSAPDTRPAPGSTAP-GST)[17]. M–FP was produced by conjugation of the FP to oxidized mannan (Sigma Chemical Co., St. Louis, MO) as previously described[12].

Adjuvants

Recombinant ovine interleukin-1β (rovIL-1β; specific activity 2×10^7 U mg^{-1}) was kindly provided by Dr A. Nash (School of Veterinary Science, The University of Melbourne, Victoria, Australia). The cytokine was obtained in a freeze dried form and reconstituted in distilled H_2O before use and administered at 0.5 μg per immunization. Aluminium hydroxide gel (alum) was prepared as described by Harlow and Lane[18] and used at one-tenth of the final immunizing volume.

Cell lines

A MUC1 expressing cell line (P815 transfectant,[19]) was obtained from Dr B. Acres (Transgene, France) and used as a source of target cells for chromium release assays. This cell line and primary spleen cell cultures were maintained in RPMI 1640 culture media (CSL, Victoria, Australia) supplemented with 10% v/v fetal calf serum (Gibco, New York), 2 mM glutamine (Gibco), 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin (Gibco), 0.12 mg ml^{-1} 2-mercaptoethanol (Sigma) and 5 mM Hepes buffer (Gibco). Cultures were incubated at 37°C in 10% CO_2.

Animals and immunizations

BALB/c mice (H-2^d) were bred at the Austin Hospital Biomedical Research Laboratory, Victoria, Australia and used at 6–8 weeks of age.

All mice were immunized i.p. and tested for antibodies (7 days after the last injection) or CTL (7 days after the last injection) or CTLp (14 days after the last injection). Mice which received both forms of antigen (FP and M–FP) were injected at opposite sites.

CTL assays

Direct CTL assays were performed 7 days after final immunizations were administered. Splenocytes were prepared from immunized mice and added to 96-well tissue culture plates in duplicate doubling dilution series, beginning with 1×10^6 cells per well. Target cells (MUC1 expressing P815 transfectants) were labelled with ^{51}Cr, washed thoroughly and added to culture plates at 1×10^4 cells per well, giving effector:target ratios of 100:1 in the first dilution well. Maximum and minimum release wells were prepared by adding target cells to 10% sodium dodecyl sulfate (SDS) or media, respectively. Plates were centrifuged and then incubated for 4 h, before removal of 80 ml of supernatant to duplicate plates containing 120 μl per well of scintillant (Microscint 40; Packard Instruments B.V., Groningen, Netherlands). Chromium release was measured using a Packard Instrument Co. (Meriden, CT) Top Count microscintillation counter and percent specific release calculated for each effector:target ratio as $100 \times$ (total release − minimum release)/(maximum release − minimum release).

CTLp assays

CTLp frequencies were calculated by limiting dilution analysis as follows. Spleen cells of immunized and naive mice were prepared for use as responder and stimulator cells, respectively. Stimulator cells were treated with 25 μg ml^{-1} mitomycin C (Kyoma, Japan) for 1.5 h and washed thoroughly in culture media before use. Serial twofold dilutions of responder cells were added to 96 well plates using 32 replicate wells at

each dilution, 100 μl per well. To each well was added 2 U recombinant human IL-2, 5 μg of Cp13-32 peptide (PAHGVTSAPDTRPAPGSTAP), and 5×10^5 treated stimulator cells in a total volume of 100 μl. Control wells (32 replicates) were prepared which lacked (a) responder cells, (b) IL-2 and peptide or (c) peptide. After 7 days incubation, 100 μl of supernatant was removed from each well and target cells added (MUC1 expressing P815 transfectants; 1×10^5 cells per well; labelled with ^{51}Cr and washed) (P815 cells non transfected were also used as targets and these were negative). Maximum and minimum release wells were prepared by adding target cells to 10% SDS, or culture media, respectively. Plates were centrifuged to maximize cell contact and incubated at 37°C for 4 h. After incubation, 100 μl of supernatant were carefully removed from each well and transferred to duplicate plates containing 120 μl per well of scintillant. Wells were considered responsive for cytotoxic activity if they yielded ^{51}Cr release counts greater than three standard deviations above the mean value obtained for all control wells. CTL precursor frequencies were determined as the inverse of responder cell dose required to generate 37% negative wells[20,21].

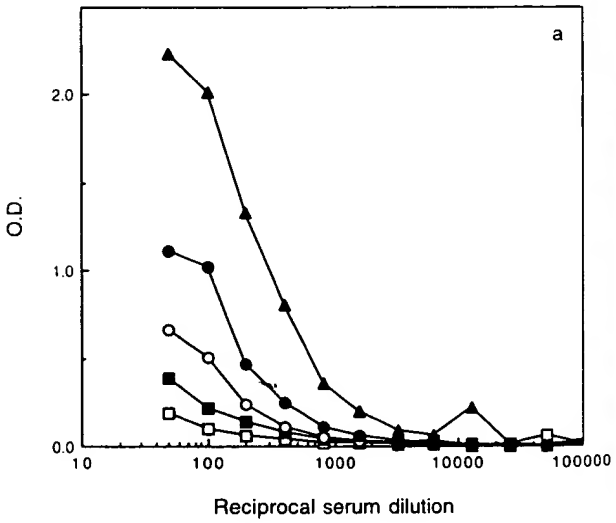
ELISA

Blood was collected from the retro-orbital sinus of mice and the serum collected after centrifugation. Serum was stored at −20°C before use. Antibody responses against the Cp13-32 peptide of human MUC1 were assessed by ELISA as previously described[22].

RESULTS

Manipulation of immune response by dose variation

To investigate the dose–response relationship for oxidized M–FP immunization, groups of mice were immunized at days 0, 10 and 17 with varying amounts of the antigen ranging from 5 to 150 μg per immunization. Blood was collected at day 20 and assayed for antibody against the MUC1 VNTR peptide Cp13-32. Group mean antibody responses are shown in *Figure 1*(a). A distinct dose–response relationship was evident with antibody titres increasing with dose. Mean 50% response titres varied from 1:80 at the lowest dose administered to 1:400 at a dose of 100 μg of M–FP. CTLp frequencies were also measured using splenocytes prepared from animals sacrificed 14–21 days after the third immunization. Results are presented in *Figure 1*(b). This figure summarizes results collected from two immunization trials. Initially CTLp frequencies were assessed using mice that had been immunized with oxidized M–FP at doses of 0.5, 1, 5, 20, 30 and 50 μg. The results show that the antigen dose which results in the induction of high CTLp frequencies, is limited to the 1–5 μg range. In these doses CTLp frequencies were ≈1:20000, while frequencies calculated from mice immunized at doses above or below 1–5 μg were as low as 1:130000, at the extremes of the dose range tested. This protocol was repeated using M–FP doses intermediate to those tested in the first experiment and CTLp frequencies for these animals fell into the expected pattern as initially defined (2 μg, 1:10000; 15 μg, 1:37500). These results demonstrate that the induction of type-1 responses by immunization with oxidized M–FP is dependent on administration of an appropriate level of antigen. *Figure 2* compares antibody and CTLp data at the various doses tested and emphasizes the reciprocal nature of the relationship between these two responses. This figure also highlights the relatively narrow dosage window for induction of CTLp responses and the dose range for which neither strong antibody or CTLp responses were observed (20–50 μg).



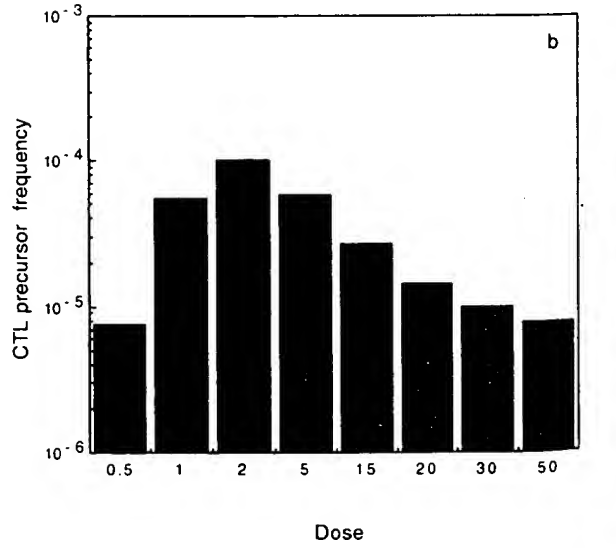


Figure 1 (a) Antibody responses against the MUC1 peptide Cp13-32 as assessed by ELISA. Titration curves shown represent mean responses from groups of five mice immunized three times with M–FP at levels of either 5 μg (□), 10 μg (■), 50 μg ( ), 100 μg (▲) or 150 μg (●) per immunization. (b) Cytotoxic lymphocyte precursor frequencies detected in spleen cell cultures from mice immunized three times with M–FP at levels ranging from 0.5 to 50 μg. CTLp assays were performed as described in the Materials and methods section. Antibody levels in non-immunized mice were negative and CTL precursor frequency was undetected

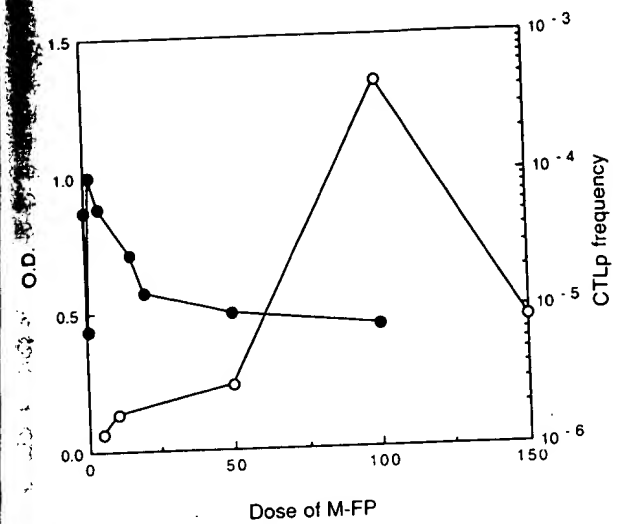


Figure 2 Comparison of CTLp and antibody responses (at 1:200 dilution) over the range of dose levels of M–FP tested. Low-dose immunization favours CTLp responses (●), while increasing the dose levels leads to a predominance of antibody response (○). A zone of relatively poor antibody and CTLp response is observed in the dose range of 20–50 μg of M–FP

Simultaneous induction of type-1 and type-2

As previous studies have shown the divergent responses induced by immunization with FP or oxidized M–FP, it was of interest to determine the phenotype of the response induced by simultaneous administration of these two forms of the MUC1 antigen. Groups of mice were immunized at days 0, 7 and 14 with a combination of 5 μg of FP and 5 μg of M–FP. Control groups were immunized with M–FP or FP only. In addition, groups of mice that had previously been immunized three times with either M–FP or FP and demonstrated type-1 or type-2 responses, respectively, were subsequently given a full course of immunization with the alternative form of the antigen. Serum was collected for antibody analysis at day 17 and CTLp assays were carried out at days 28–35. Direct CTL assays were also performed at day 21 on a sample of mice from each group.

Group mean antibody responses determined from sera of these mice are shown in *Figure 3*(a). As for results reported in previous studies, low antibody responses were recorded for mice immunized with M–FP only (mean 50% response titre of 1:200), while FP-immunized mice showed a mean titre over 100-fold higher than for the M–FP group. Animals immunized simultaneously with M–FP and FP showed antibody titres equivalent to those immunized with FP alone, as did the group that received three administrations of M–FP after an initial full course of FP immunizations. In the converse situation, when mice were immunized with FP after an initial full course of M–FP, antibody titres were improved more than tenfold over responses of mice immunized with only M–FP.

Figure 3(b) shows mean CTLp frequencies of immunized mice. In accordance with previous studies, FP immunized mice exhibited low CTLp frequencies with a mean of 1/85000, while in contrast, M–FP immunized mice showed a strong CTLp response, with a mean frequency of 1/9000. All other experimental groups showed mean CTLp frequencies similar to those of mice immunized with M–FP alone. The strongest response occurred in mice that received simultaneous administration of both forms of the MUC1 antigen (mean frequency 1/5800), and equivalent frequencies were recorded for the groups administered with the two forms of antigen alternatively (FP then M–FP; 1/10500, M–FP then FP; 1/10000). Results of the direct CTL assays are shown in *Figure 4*, and these results are in accordance with the CTLp study; only mice immunized with FP alone showed poor CTL responses. Mice that received FP followed by M–FP showed an intermediate level of CTL

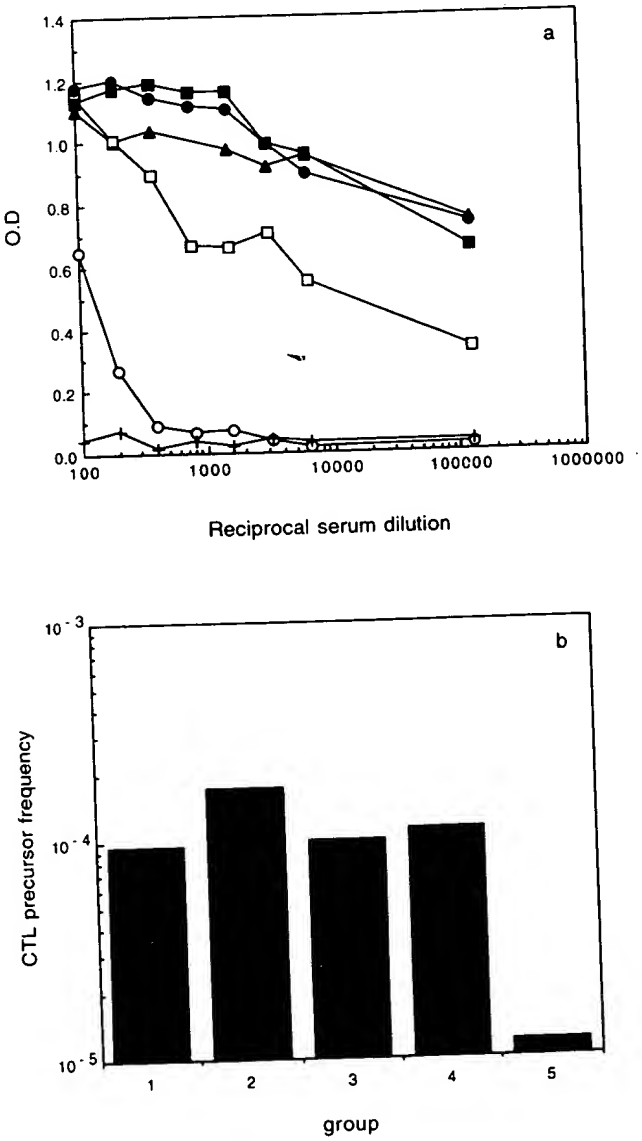


Figure 3 (a) Antibody responses against MUC1 peptide Cp13-32 as assessed by ELISA. Titration curves represent group mean responses of non-immunized mice (+) or mice immunised three times with either M–FP (○), FP (●) or combined M–FP and FP (▲), or immunised three times with MFP then three times with FP (□) or the converse (■). (b) Cytotoxic lymphocyte precursor frequencies detected in spleen cell cultures from mice immunized three times with either M–FP (1) or combined M–FP and FP (2), or three times with M–FP, then three times with FP (3) or the converse (4), or FP alone (5)

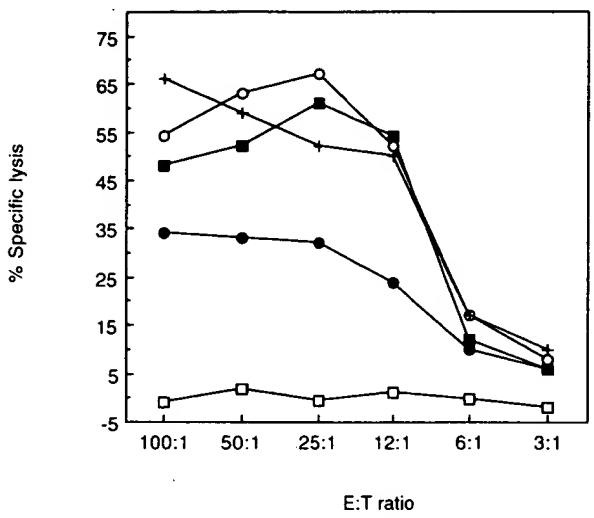


Figure 4 Cytotoxic lymphocyte assay using spleen cells of mice immunized three times with either M-FP (○), FP (□), M-FP and FP (+), or three times with M-FP, followed by three immunizations with FP (●) or the converse (■)

response, inducing 33% specific lysis at E:T of 25:1, which was approximately twofold lower than the strong CTL responses observed in the three remaining experimental groups.

Taken together, these results confirm previous reports in which a marked divergence in the type of immune response can be generated by immunizing with FP (inducing antibody responses) or M-FP (inducing CTL activity), and demonstrate that the two forms of response are not necessarily mutually exclusive. A combination of antibody and cellular responses was generated by immunization with FP and M-FP, and one form of response was superimposed on a previously generated response by administering each form of the antigen alternatively.

Use of interleukin-1β as adjuvant

It was next determined whether the response to M-FP could be modified by co-administration of a cytokine adjuvant. Groups of mice were immunized with either M-FP, M-FP plus rovIL-1β or M-FP plus rovIL-1β plus alum and immune parameters assessed as for previous experiments. Antibody responses are illustrated in *Figure* 5(a) and show that antibody titres to M-FP could be enhanced by up to fourfold by co-administration of rovIL-1β, either in combination with alum or in the soluble form. Mean CTLp responses for each group were not significantly different and equivalent to experiments described above in which M-FP was administered (*Figure* 5(b)). RovIL-1β, while increasing antibody responses, had no adverse effect on the cellular response induced by M-FP.

DISCUSSION

Divergent responses to the human mucin-1 breast cancer antigen have been previously described[11,12]. Administration of the FP incorporating five repeats of the 20 amino acid VNTR sequence results in strong antibody responses of the IgG1 isotype, a lack of CTL responses, low CTLp frequencies and little protection after challenge with MUC1 expressing tumour cells. Further, spleen cell cultures from FP immunized mice secreted IL-4, but not IFNγ into the supernatant. DTH responses (primarily $CD4^+$ T cell mediated) could be demonstrated after footpad immunization but were shown to be irrelevant to the protective responses against $MUC1^+$ tumours. Similar results were obtained when FP, conjugated to mannan under reducing conditions, was used as the antigen. In contrast, administration of M-FP, produced by conjugation of FP to the carbohydrate mannan under oxidizing conditions induces a primarily cellular response that is mediated by $CD8^+$ T cells. Antibody responses are minimal, CTL

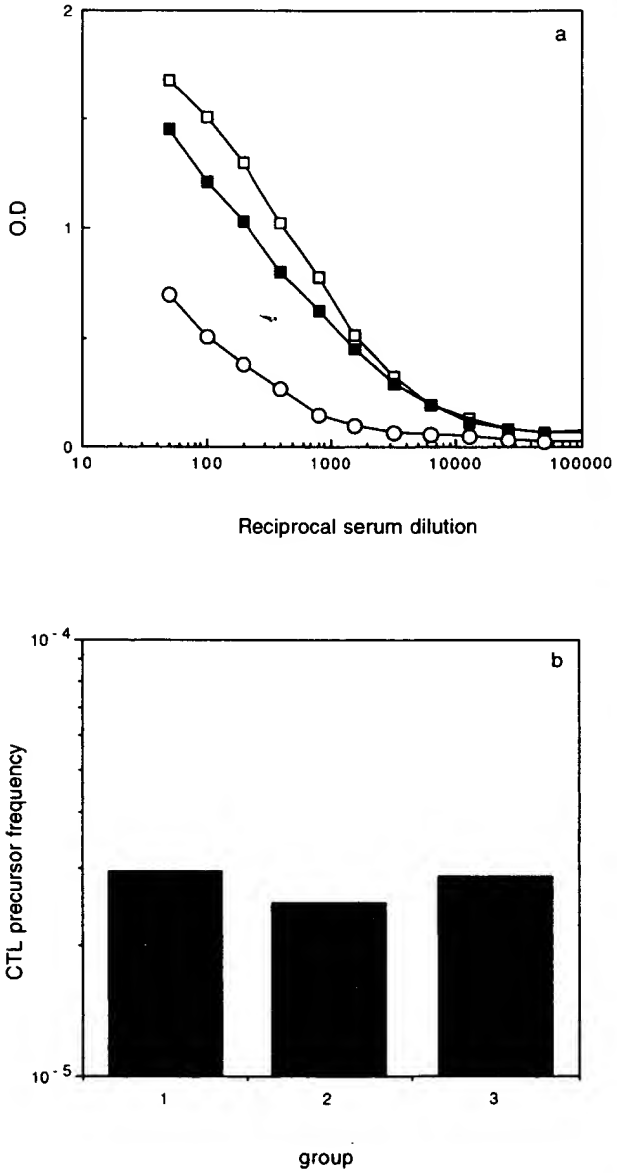


Figure 5 (a) Antibody responses against MUC1 peptide Cp13-32 as assessed by ELISA. Titration curves represent group mean responses of five mice immunized three times with either MFP (○), MFP and rovIL-1β (■) or M-FP and rovIL-1β and alum (□). The dose of M-FP injected was 5 μg. (b) Cytotoxic lymphocyte precursor frequencies detected in spleen cell cultures from mice immunized three times with either M-FP (1) or M-FP and rovIL-1β (2) or M-FP and rovIL-1β and alum

responses strong, and high CTLp frequencies are induced. Spleen cell cultures secreted IFNγ and not IL-4, antibody responses were predominantly of the IgG2a isotype and DTH responses were again observed. This response induces complete protection at challenge. These divergent responses to M–FP and FP fit the pattern of type-1 and type-2 responses, respectively, and results described in this report for separate immunization with either of these two forms of the antigen confirm these previously described results.

In experiments described here the induction of the type-1 response through administration of M–FP was dependent on selection of an appropriate dose of antigen. Outside the optimal dose range, responses to M–FP began to adjust towards a type-2 response in a dose dependent manner. Similar dose–response studies using FP alone showed no alteration in the phenotype of the ensuing immune response (results not shown). The effect of antigen concentration or chemical modification of the antigen, on the modulation of the ensuing immune response has frequently been reported[7]. Janeway *et al.*[23] described low density antigen presentation as a factor promoting the development of type-2 responses, while increasing antigen concentration induced a shift towards a type-1 response. Contrasting results were described by Bretscher *et al.*[24] using a *Leishmania major* mouse model. BALB/c mice, 'innately susceptible' to the parasite due to their tendency to produce non protective antibody responses to infection, are capable of mounting cellular immune responses to the parasite when immunized with only a small number of parasites. Subsequently, it has also been proposed that tuberculosis and leprosy vaccines could be optimized by reduction of the dose of antigen administered, resulting in induction of improved cellular responses required for protection against these intracellular parasites[25]. Although these reports did not also note a shift towards type-2 responses at extreme low antigen doses as described for M–FP administration, *in vitro* studies by Hosken *et al.*[26] demonstrated that both high and low dose stimulation of $CD4^+$ cells isolated from DO11.10 TCRab transgenic mice induce type-2-like responses, while at moderate doses type-1 responses occur, as determined by secretion of cytokines in restimulated cultures.

When delivered at an appropriate dose level, M–FP is indeed capable of inducing an immune response distinct from, and opposite to, that induced by administration of the unconjugated fusion protein. Several factors that have been shown to affect the development of divergent T cell responses: Although the cytokines present during the early phase of the immune response exert a major influence on T cell differentiation, factors inducing the secretion of these cytokines may include antigen concentration (described above), antigen presenting cell type[27], the nature of the antigen[28–30], route of administration[31,32] or the quality of the MHC:TCR binding[33]. Responses to M–FP described here are consistent with the observation that the type-1 phenotype is favoured after antigen presentation by macrophages[34], assuming that macrophages selectively bind the M–FP antigen via their mannose receptor. Recently it was shown that dendritic cells also express the mannose receptor[35], although it is not clear if the mannose receptor on the dendritic cells is the same as the mannose receptor on macrophages. The mechanism of action is currently under investigation.

The experiments described here however, demonstrate that type-1 and type-2 responses to M–FP/FP are not mutually exclusive and may be induced simultaneously, or one response may be induced subsequent to the other. In most situations, the immune response to infection or immunization involves both cellular and humoral components: if the antigenic challenge is strong and persistent, there is a greater likelihood of a distinct polarization of responses. In our experiments, polarization into type-1 or type-2 responses was observed after the initial three-dose immunization regime, but a second, different response could be superimposed, with no apparent antagonistic effect on the original response. Although a combination of cellular and humoral responses could arise under the influence of T helper cells (Th-0) or $CD8^+$ T cells that are able to secrete both IFNγ and IL-4, results described here also suggest that cells that typically secrete these cytokines independently (type-1/type-2) have combined activity in producing the responses observed. Given the number of factors that have been shown to influence the development of T cell responses, it is not surprising that administration of antigen in two different forms can result in expression of both cellular and humoral responses. Temporal and spatial differences in secretion of these cytokines in response to the two forms of antigen may allow the coexpression of cytokines that are generally considered to be antagonistic; recent studies have shown that FP is diffusely distributed whereas M–FP localizes preferentially to the liver and spleen after injection, supporting this premise (V. Apostolopoulos, unpublished data).

Experiments that used rovIL-1β as an adjuvant for M–FP immunization support our findings that the antibody response may be enhanced without inducing an antagonistic effect on cellular responses. The adjuvant action of IL-1 has been previously observed when the cytokine was administered in conjunction with both model antigens[36–38] and experimental vaccines[39–41] with the primary effect being enhancement of antibody responses. Low doses of IL-1 used to induce adjuvant activity do not induce inflammatory responses[37,42]. The converse effect has recently described when IL-12 was used as an adjuvant for immunization with TNP–KLH[43]: this cytokine enhanced type-1 responses without adversely affecting type-2 responses, supporting the concept that cellular and humoral responses can be manipulated independently. These findings are supported by studies that have shown that many cytokine genes are independently regulated[44].

This report demonstrated that type-1 and type-2 immune responses against MUC1 antigens are not mutually exclusive and can develop simultaneously. The administration of a combination of antigen forms to develop a mixed immune response would be beneficial for prophylactic vaccination in situations where both types of response are required for complete resolution of infection. Viral infection, requiring clearance of both free virus and infected cells is the most obvious example. In addition, results reported here describing development of one immune phenotype subsequent to another shows that thera-

peutic immunization in the presence of a pre-existing immune response may alter the response towards one more appropriate for resolution of infection.

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